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# Estimation of reference curves for the urinary steroid metabolome in the first year of life in healthy children: tracing the complexity of human postnatal steroidogenesis

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## Graphical abstract

### Highlights

The steroid metabolome during fetal-neonatal transition varies with sex and postnatal age.

For 67 urinary steroid compounds normative values have been quantified by GC-MS for the first year of life.

Highest concentrations are found for progesterone metabolites and glucocorticoids.

In both sexes, most compounds peak at week 3 and decrease to 20% by week 25.

Normative data for the urine steroid metabolome provide a powerful diagnostic tool.

### Abstract

**Context:** Complex steroid disorders such as P450 oxidoreductase deficiency or apparent cortisone reductase deficiency may be recognized by steroid profiling using chromatographic mass spectrometric methods. These methods are highly specific and sensitive, and provide a complete spectrum of steroid metabolites in a single measurement of one sample which makes them superior to immunoassays. The steroid metabolome during the fetal-neonatal transition is characterized by a) the metabolites of the fetal-placental unit at birth, b) the fetal adrenal androgens until its involution 3-6 months postnatally, and c) the steroid metabolites produced by the developing endocrine organs. All these developmental events change the steroid metabolome in an age- and sex-dependent manner during the first year of life.

**Objective:** The aim of this study was to provide normative values for the urinary steroid metabolome of healthy newborns at short time intervals in the first year of life.

**Methods:** We conducted a prospective, longitudinal study to measure 67 urinary steroid metabolites in 21 male and 22 female term healthy newborn infants at 13 time-points from week 1 to week 49 of life. Urine samples were collected from newborn infants before discharge from hospital and from healthy infants at home. Steroid metabolites were measured by gas chromatography-mass spectrometry (GC-MS) and steroid concentrations corrected for urinary creatinine excretion were calculated.

**Results:** 61 steroids showed age and 15 steroids sex specificity. Highest urinary steroid concentrations were found in both sexes for progesterone derivatives, in particular 20 $\alpha$ -DH-5 $\alpha$ -DH-progesterone, and for highly polar 6 $\alpha$ -hydroxylated glucocorticoids. The steroids peaked at week 3 and decreased by ~80% at week 25 in both sexes. The decline of progestins, androgens and estrogens was more pronounced than of glucocorticoids whereas the excretion of corticosterone and its metabolites and of mineralocorticoids remained constant during the first year of life.

**Conclusion:** The urinary steroid profile changes dramatically during the first year of life and correlates with the physiologic developmental changes during the fetal-neonatal transition. Thus detailed normative data during this time period permit the use of steroid profiling as a powerful diagnostic tool.

**Keywords:** newborn infants, gas chromatography-mass spectrometry, urinary steroid profile, reference values

## 1 Introduction

Steroid hormones regulate various biological processes including salt balance, sexual development, reproductive function, and immune and stress responses. Because the secretion of each steroid hormone may vary with age and sex, especially in the newborn period during adjustment to extra-uterine life and fetal adrenal involution, detailed normative data are needed for effective quantitative assessment of steroids.

For the urine metabolome during fetal-neonatal transition, Shackleton [1] has defined three groups: 1) steroid metabolites derived from the fetal-placental unit which are expected to vanish off rapidly in the newborn after birth; 2) metabolites produced by the fetal adrenal zone which should decrease parallel to its involution during the first 3-6 months after birth; 3) steroid metabolites derived from the developing endocrine cells and organs of the growing newborn. During fetal life steroids are mainly produced in the fetal zone of the adrenal glands, the testes (the ovaries being quiescent), the placenta and the brain [2, 3]. Postnatally, the fetal adrenal, which abundantly produces 19-carbon (C19) steroids (DHEA, DHEAS, androstenedione), involutes within 3-6 months [4]. After birth the adult adrenal cortex in its zonae glomerulosa and fasciculata produces mineralocorticoids and glucocorticoids. As adjusted for body surface area, cortisol secretion is essentially constant throughout postnatal life and shows little sex difference (except during pregnancy, when cortisol secretory rates increase), whereas aldosterone concentrations are very high in the newborn, drop rapidly during the first year, then continue to fall during childhood [4, 5]. The zona reticularis of the adult adrenal cortex is formed slowly during infancy to become active in C19 steroid production during adrenarche at 7-10 years. Testicular steroidogenesis is very active in the production of androgens in the male fetus, then declines after birth with a temporary increase during minipuberty between postnatal days 30-100 before going into the quiescent prepubertal phase [5]. By contrast, estrogens are low from infancy until puberty as the prepubertal ovary is quiescent [4, 5].

Steroid hormones can be grouped according to their receptor binding into progestins, androgens, estrogens, mineralocorticoids and glucocorticoids. The biochemistry of steroid biosynthesis and metabolism is largely known and specific steroid pathways are regulated by differential expression and activity of enzymes and cofactors involved in a developmental, sex, time and tissue specific fashion which might be perturbed in disease states [4, 5]. Once steroids are produced in steroidogenic tissues, they enter the circulation and are converted into a large number of metabolites by the liver and peripheral tissues before being excreted in the urine. Thus, all these processes contribute towards the steroid metabolome, which may be characterized from biomaterials such as blood or urine, and serves as an excellent diagnostic tool.

In the past, some effort has been made to generate quantitative data for the urinary steroid metabolome in newborns (for an overview see *Appendix Table A*) [1, 6-11]. However, detailed normative data are lacking for the first year of life when many changes happen over short time intervals in healthy babies; normative data are also needed to distinguish physiologic alterations from inborn errors of metabolism in sick babies. Simultaneous measurements of the full spectrum of urinary steroids by GC-MS provide a comprehensive and integrated picture of the steroid metabolome, permitting accurate diagnosis of disorders of steroid biosynthesis and metabolism in newborn infants [12]. In addition, the non-invasive sampling and the requirement of small amounts of spot urine make this method very suitable for infants and small children.

We intended to describe and quantitate the urinary steroid metabolome of healthy infants longitudinally at high resolution time intervals during the first year of life using GC-MS. We analyzed the 67 measured steroids for sex differences and longitudinal changes that might indicate underlying developmental processes for each measured steroid and for steroid groups. Our study now provides detailed normative data in percentile curves for 67 steroid metabolites during the first year of life. We show that there are few sex-specific differences but that there are significant longitudinal changes that relate to developmental changes in steroidogenesis during fetal-neonatal transition.

## **2 Material and Methods**

### **2.1 Study population**

The local medical ethics committee approved the study protocol, and the parents provided written informed consent. Healthy newborn infants and their parents were recruited from June 2010 to March 2012 at the University Children's Hospital Bern, Inselspital, the Lindenhofspital Bern and the Spital Münsingen, Bern, Switzerland. Inclusion criteria were: Caucasian healthy boys and girls, born at term with normal weight and length, either by vaginal delivery or Caesarian section, willingness of the parents to collect 13 urine samples throughout the first year of life and to report on the health status of their baby (including weight and length at each urine sampling timepoint). We recruited 43 subjects (22 girls and 21 boys) and obtained samples at 13 time points, for an anticipated total of 559 samples. Drop-outs were due to the following reasons: stop of sampling or non-compliance with sampling schedule, occurrence of disease and drug treatments or errors in measuring steroids and/or creatinine.

### **2.2 Urine collection procedure**

Urine samples were collected from each study subject in weeks 1, 3, 5, 7, 9, 11, 13, 17, 21, 25, 33, 41, and 49 of life if healthy and receiving no medication. A maximal deviation of  $\pm 7$

days from this time schedule was tolerated, except for week 1 where a deviation of only  $\pm 3$  days was permitted. Clean spot urines were collected from pure cotton balls inserted into regular diapers. Wet cotton balls, free of stool, were removed and urine was pressed out with a 50 ml syringe into a labeled tube. Samples were stored at  $-20^{\circ}\text{C}$  and were subsequently sent to the steroid laboratory of the Department of Nephrology, Hypertension and Clinical Pharmacology at the University Hospital of Bern, Switzerland, for GC-MS steroid analysis. Validity and stability of this urine collection method for steroid analysis has been previously reported [13-15]. Minimal urine volume required for steroid analysis was 200  $\mu\text{l}$ , standard volume was 1.5 ml; for creatinine measurement 5  $\mu\text{l}$  urine was used.

### 2.3 Measurement of urinary steroid metabolites and creatinine

Quantitative analysis of 67 different urinary steroid hormone metabolites was performed by an *in-house* adapted GC-MS method as previously described [16-19]. All measurements were performed in the same steroid laboratory of the Department of Nephrology, Hypertension and Clinical Pharmacology at the University Hospital of Bern, Switzerland. Measured steroids are listed in Table 1 and depicted in Figure 1.

Standards, reagents and enzymes were obtained as follows: The steroids medroxyprogesterone, stigmasterol, and  $3\beta 5\beta$ -TH-aldosterone were obtained from Steraloids (Newport RI, USA), the Sep-Pak C18 column from Waters AG (Baden-Dättwil, Switzerland), the acetate buffer from Merck (Zug, Switzerland), the powdered sulfatase enzyme originated from *Helix pomatia* from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland), the  $\beta$ -glucuronidase/arylsulfatase liquid enzyme from Roche Diagnostics AG (Rotkreuz, Switzerland), the derivatives methoxyamine HCl 2% and the trimethylsilylimidazole (TMSI) from Thermo Scientific (Waltham MA, USA), the pyridine from company (city, country), the Lipidex 5000 from Perkin Elmer (Waltham MA, USA).

In brief, the urine sample preparation consisted of pre-extraction, enzymatic hydrolysis, extraction from the hydrolysis mixture, derivatization and gel filtration. Steroid profiles data were obtained by adapted GC-MS analysis according to methods reported by Shackleton [16]. In detail, the preanalytic procedures were as follows: Step 1, to 1.5 ml of urine 2.5  $\mu\text{g}$  of medroxyprogesterone was added as a recovery standard. Step 2, the sample was extracted by solid phase extraction on a Sep-Pak C18 column, dried under nitrogen and reconstituted in 0.1 M acetate buffer (pH 4.6). Step 3, the sample was hydrolyzed with 15 mg of powdered sulfatase enzyme and 15  $\mu\text{l}$  of  $\beta$ -glucuronidase/arylsulfatase liquid enzyme before step 4, the free steroids were again extracted on a Sep-Pak C18 cartridge. During step 5, a mixture of standards for chromatography and derivatization consisting of 2.5  $\mu\text{g}$  of Stigmasterol and 0.15  $\mu\text{g}$  of  $3\beta 5\beta$ -TH-aldosterone was added to the extract. Step 6, 100  $\mu\text{l}$  of methoxyamine HCl 2% in pyridine was added and the samples were heated at  $60^{\circ}\text{C}$  for one hour. Step 7, after evaporation of the solvent 100  $\mu\text{l}$  of trimethylsilylimidazole (TMSI) was added and the

extracts were heated at 100°C for 16 hours. Step 8, the methyloxime-trimethylsilyl ethers derivatized samples were purified by gel filtration on Lipidex 5000 columns to remove the excess of derivatization reagent. In three samples, the urine volume available was 200 µl only. For that case and future use, we re-validated our method for reduced sample volume and give all data of intra- and inter-assay variations for standard and reduced volume in Appendix Table B.

Mass spectrometric analyses were performed on a gas chromatograph 7890A from Agilent Technologies (La Jolla, California, USA) coupled to a mass selective detector Hewlett-Packard 5975C providing selected ion monitoring (SIM). For each compound measured, one characteristic ion was monitored. The derivatized samples were analyzed during a temperature programmed run (210-265°C) over a 35-minute period. A mixture containing a known amount of all steroids measured was analyzed on a regular basis to act as a calibration standard. Recoveries were checked with medroxyprogesterone and corrections were made for the losses occurring during sample preparation. In Appendix Table C the steroid metabolites measured in this study and their molar mass, each selected compound-specific qualifier ion, the retention time and the limits of detection and of quantitation are given. Due to lack of reference materials, the five compounds 6 $\alpha$ -OH-TH-cortisone, 1 $\beta$ -OH-TH-cortisone, 1 $\beta$ -OH- $\beta$ -cortolone, 6 $\alpha$ -OH- $\alpha$ -cortolone and 6 $\alpha$ -OH- $\beta$ -cortolone were calibrated and measured according to the procedure published by Caulfield et al. [8]. Neonatal urine from the first collection after birth containing high amounts of these steroids was analyzed by GC-MS in the scanning mode and the total ion current (TIC) peak areas of the five identified compounds were integrated and were related to the standard Stigmasterol. Based on the assumption that peak area responses in TIC of different compounds were identical for components of similar retention time, we determined the approximate concentration of these compounds in the urine and they were finally analyzed again in the SIM mode to obtain calibration responses which were used to quantify the five compounds in the study samples. For all steroids, measured specific peaks in the chromatograms had to be three times higher as the noise above the baseline to be counted as valid measurements (signal-to-noise-ratio  $\geq 3$ ).

Urinary creatinine was measured by quantitative colorimetry using the QuantiChrom Creatinine Assay according to the manufacturer's recommendation (DICT-500; BioAssay Systems, Hayward, CA, USA).

Since spot urines were collected, measured steroids were standardized by urinary creatinine concentration and expressed in µg/mmol creatinine [20, 21].

## 2.4 Quality control of the applied GC-MS method

The reproducibility of our *in-house* GC-MS method is continuously monitored by an internal quality control. For this purpose 15 samples of frozen aliquots from the urine of a healthy



volunteer with an unremarkable urinary steroid profile were measured and means, standard deviations, and the coefficients of variation for all steroid metabolites were calculated as standard values. Two samples of this urine served as internal quality control in all measurement series and the results of the two analyses were regularly compared with the standard values.

Since 10 years we and 27 other laboratories from all over the world participate regularly in an external quality control organized by the University College London Hospitals (London, United Kingdom) and by the Foundation for Quality Medical Laboratory Diagnostics skml (Stichting Kwaliteitsbewaking Medische Laboratoriumdiagnostiek, Nijmegen, The Netherlands). For that, at the beginning of each year every laboratory receives 12 lyophilized urines, of which a specific one is dissolved every month in water and analyzed like a patient sample. The results of the analysis are sent together with an interpretive comment, a proposal for further investigations and a chromatogram to the reference laboratory. The individual urinary steroid values of each laboratory are compared with the median value of all participants, the multiple of the median (MoM) is calculated for each steroid metabolite and the average MoM is given for each laboratory. Each laboratory gets a score for the analytical results as well as for the comment and the chromatogram. At the end of the year, a ranking for the steroid analysis, for the comment and an overall ranking is calculated for all 28 laboratories by the organizers.

## 2.5 Statistical analysis

Baseline characteristics of the study population are presented as mean, standard deviation, median and range unless otherwise stated. All normalized urinary steroid hormones were modeled on the logarithmic scale, where the variance was roughly constant across the ages. The age and sex dependent mean of each log-steroid was estimated using a linear mixed model which included a random subject effect to account for the dependence induced by the repeated measurements within the subject. To allow a flexible modelling of the age-dependence of various steroids, we considered the following six possible age-effects: constant (corresponding to no age effect), linear, quadratic, quadratic spline with one knot, quadratic spline with one knot and a constraint of levelling-off, quadratic spline with two knots and a constraint of levelling-off. The knots have been selected using a grid search in order to optimize the fit, while a constraint of levelling-off, allowing to model a plateau reached after some age, can be easily imposed using B-splines [22-24]. Since we considered to include a sex effect in the model, we fitted a total of 12 possible models for each steroid and selected that model minimizing the Akaike Information Criterion. We let thus the data decide whether or not an age or a sex effect should be included in the model. The detailed model selection process is described in *Appendix Methods B*. Normative values of the study population were fitted from the model that was finally selected and are

presented as the 10<sup>th</sup>, 50<sup>th</sup> and 90<sup>th</sup> percentiles and curves for each steroid. Data are presented for steroid groups and time-points as well as separately for male and female infants when a model with a sex effect was selected. Steroid hormones were grouped according to their receptor binding into progestins, androgens, estrogens, mineralocorticoids and glucocorticoids. As corticosterones can bind to both the mineralocorticoid receptors and the glucocorticoid receptors we decided to form a separate group for them. Inactive steroid metabolites which do not bind to a receptor (e.g. 17-hydroxyprogesterone and the progesterone receptor), were included in the group of steroids of the most proximal active precursor steroid (e.g. group of progestins for 17-hydroxyprogesterone). Statistical analyses were performed using the software R including the lme4 package [25].

### 3 Results

#### 3.1 Study population and specimen characteristics

Serial urine samples of 21 healthy boys and 22 healthy girls were available for analysis. The baseline characteristics of the study population stratified by sex at birth and at 1 year of age are presented in **Table 2**. From the recruited 43 subjects and 13 time-points, 26'914 values of creatinine corrected steroid compounds were included in the final analysis corresponding to 71.9 % of the maximal possible number of calculated values.

#### 3.2 Urinary steroid metabolome

67 steroids (Figure 1 and Table 1) were assessed in each urine portion by GC-MS in 43 babies at 13 time-points from birth to one year of age and tested for sex specificity and age-related changes. Six of 12 possible models described in the method section allowed the best estimate of the log-steroid mean for characterization of all steroids.

Two steroids, TH-aldosterone and 17 $\alpha$ -20 $\alpha$ -DH-progesterone, showed no association with age or sex. Four steroids, 11 $\beta$ -OH-progesterone, androstenediol, 18-OH-11-dehydro-TH-corticosterone and 18-OH-cortisol showed an association with sex but not with age. An association with sex was found for 15 steroids. Age-related changes were observed in 61 steroids, 50 within the group of steroids not specific for sex and 11 in the group of sex specific steroids. The 10<sup>th</sup>, 50<sup>th</sup> and 90<sup>th</sup> percentiles estimated for urinary steroids unassociated with sex are given in Table 3, and in Table 4, if sex-specific differences were detected. In both tables the association with age is also indicated.

The median concentration of total excreted steroids at weeks 1, 3, 5 and 7 was estimated at 29'379-36'906  $\mu$ g/mmol creatinine in males and 20'959-26'330  $\mu$ g/mmol creatinine in females (Figure 2A, B). A substantial decline in the excreted steroids then occurred in both sexes followed by constant excretion from weeks 33 to 49 in males (median concentration 6'780  $\mu$ g/mmol creatinine) and females (median concentration 4'837  $\mu$ g/mmol creatinine).

Group analyses of steroid metabolites showing age- and sex-specific characteristics are shown in Figure 2C-L. A model with a sex effect but without an age effect was selected for corticosterones (Figure 2G, H), a model with an age and sex effect was selected for glucocorticoids (Figure 2J, K) and the total sum of steroids (Figures D, E), and a model with an age effect but without a sex effect was selected for progestins, androgens, estrogens and mineralocorticoids (Figure 2C, F, I and L). The resulting estimated percentiles fit the data nicely for most of the steroids and steroid groups, as can be seen in Figure 1 and the *Appendix Fig. C.1-C.82*, where the numbers of observations falling below, between and above the estimated 3<sup>rd</sup>, 10<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup>, 90<sup>th</sup> and 97<sup>th</sup> percentiles were close to the theoretical values. Androgens and estrogens were markedly higher at birth and declined during the first 6 months, but the two measured estrogen metabolites were very low, even during the first week of life, when compared to androgens such as testosterone or 5 $\alpha$ -DH-testosterone. Similarly, progestins declined within 5-6 months after birth and remained constant thereafter. At week 1 glucocorticoids and progestins each contributed about one third to the total amount of excreted steroids in both males and females whereas androgens contributed about 15%. While the proportion of progestins and androgens declined during the first weeks of life, the proportion of excreted glucocorticoid metabolites increased constantly until week 21 when it comprised about 80% of the total amount of the excreted metabolites. The steroid metabolites with the highest urinary concentration in both sexes at nearly all time-points were 20 $\alpha$ -DH-5 $\alpha$ -DH-progesterone among the group of progestins, 6 $\alpha$ -OH- $\alpha$ -cortolone among the glucocorticoids, 5-androstenetriol among the androgens, estriol among the estrogens, and 11-dehydro-TH-corticosterone among the corticosterones (Table 3 and 4).

#### 4 Discussion

The recent discovery of novel, complex steroid disorders such as P450 oxidoreductase deficiency or apparent cortisone reductase deficiency highlight the diagnostic significance of the steroid metabolome [26-28]. Also, many now propose that steroids should no longer be measured by inaccurate immunoassays and that steroid assays should be replaced by mass spectrometric methods, which are highly specific and sensitive, while providing a full spectrum of steroid measurements in a single sample in one assay [29]. Although standard methods for steroid profiling from urine or plasma are known, they are only established in few laboratories and normative data are scarce. We have been using GC-MS for steroid profiling from urine for the past 25 years in our laboratory but did not have detailed normative data for neonates and infants in the first year of life. Although some normative data from other laboratories exist, data for the first year of life, when dramatic changes occur, are incomplete. Such normative data may not be applied for individual *in-house* methods without proper controls and are difficult to obtain. Therefore, to generate a comprehensive picture of

the steroid metabolome during early development, we measured 67 urinary steroids in 21 male and 22 female full-term healthy infants at 13 different time-points from week 1 to week 49 of life by GC-MS and calculated normative data using specifically developed mixed-linear models for each steroid.

We used a statistical model that includes a flexible age-dependence to capture possible pattern of development among the various steroids. This model uses a limited number of variables to characterize the distribution of steroids at a given age, yielding a robust estimation of the high percentiles. We obtained explicit, flexible, smooth and robust age-dependent and sex-dependent percentiles that were as close as possible to our data. With our steroid normative data percentiles we provide an easy tool for clinical use.

Our steroid profiling results may be directly compared to previous reports of creatinine-corrected urinary steroid metabolites; these are summarized in *Appendix Table A*. In particular, comparison is possible with the most comprehensive collection of urinary steroid metabolites so far assessed by Homma et al. who measured 50 steroid metabolites in 18 babies aged 5-22 days [9]; of those 24 metabolites were also included in our study. We found similar results for 16 urinary steroids in the first weeks of life, specifically 17-OH-pregnenolone, pregnanetriol, pregnanediol, androsterone, dehydroepiandrosterone, 11 $\beta$ -OH-androsterone, 16 $\alpha$ -OH-dehydroepiandrosterone, estradiol, 18-OH-11-DH-TH-corticosterone, TH-aldosterone, TH-11-deoxycortisol, cortisone, TH-cortisone,  $\alpha$ -cortolone, cortisol and 18-OH-cortisol [9]. Values more than 3-fold higher were found in our study for pregnanetriolone, pregnanediol, etiocholanolone, androstenediol, estriol, and 20 $\alpha$ -DH-cortisol while lower values were found for 20 $\alpha$ -DH-cortisone and 20 $\beta$ -DH-cortisone. Similar to our study, Homma et al. also found no sex difference for most of the urinary steroid metabolites including the majority of the androgens [9]. Like Homma's study, we found a sex difference for progesterone metabolites, but unlike Homma's study, we found no sex difference for estradiol and estriol, and we found considerably higher estriol concentrations. These differences might be explained by technical limitations when measuring low levels. On the other hand, some of the observed differences may also be explained by differences in the genetic background of studied subjects affecting the steroid metabolome; e.g. while we studied Swiss infants, Homma et al. studied Japanese.

We compared our steroid data concerning sex- and age-specificity to previous studies assessing 24 hour urinary excretion of steroid metabolites in the first year of life [10, 11]. Concerning sex-specificity, our results are in line with both published reports with the exception of allo-TH-cortisol, for which we found about 2-fold higher values in male infants during the first year of life similar to the study of Wudy et al [10], but different to the values reported by Rogers et al. [11]. Concerning age-specificity, we also found similar patterns of

urinary steroid excretion compared to Rogers et al. who demonstrated an increase for most cortisol metabolites during the first year of life [11]. However, we could not confirm the increase of total measured cortisol metabolites across the first year of life as described by Rogers et al. But this difference might be best explained by the fact that total glucocorticoid excretion consisted of 9 metabolites in Rogers' study but 21 metabolites in ours. Thus the group results cannot be directly compared. Our glucocorticoid group analysis in particular included five highly polar  $1\beta$ - and  $6\alpha$ -hydroxylated steroids (namely  $1\beta$ -OH-TH-cortisone,  $1\beta$ -OH- $\beta$ -cortolone,  $6\alpha$ -OH-TH-cortisone,  $6\alpha$ -OH- $\alpha$ -cortolone and  $6\alpha$ -OH- $\beta$ -cortolone), of which specifically  $6\alpha$ -OH- $\alpha$ -cortolone was highest at birth, increased even further in the first weeks, before slightly decreasing and leveling off after week seven.

Unlike urinary cortisol metabolites, we found only few published data on progesterone metabolites (progestins) to compare our study results (Table 3 and 4 and *Appendix Table A*). Our study provides data on 26 progestins, of which only normative data of five were reported and are in accordance with our values [8-10]. The highest measured progesterone metabolite in our study was  $20\alpha$ -DH- $5\alpha$ -DH-progesterone in both sexes from birth to one year of age. For this metabolite we found no measurements in the literature and its function is also not described. It might be that this is just the major highly polar metabolite to excrete progestins effectively without other function. Similar to our findings for cortisol metabolites, we found high urinary concentrations of polar  $6\alpha$ -hydroxylated progesterones, in particular  $6\alpha$ -OH-progesterone and  $6\alpha$ -OH- $3\alpha5\beta$ -TH-progesterone, which were excreted at concentrations above 100  $\mu\text{g}/\text{mmol}$  creatinine until week 13, that is much higher than most other progestins.

In comparison to glucocorticoids, androgens and estrogens are excreted in considerably smaller amounts and decrease in the first months of life. This is explained by the underlying developmental changes of the steroid organs as described earlier (see Introduction).

Overall, our data are largely in line with published results but expand the picture of the steroid metabolome of the first year of life by giving a higher resolution and more metabolites. Our data also confirm that the urinary steroid metabolome of a newborn can track normal and abnormal developmental processes of steroid organs and therefore provides an excellent, non-invasive diagnostic tool.

The reference ranges published in this study will raise the question about their usefulness for other laboratories using slightly different methods. We are convinced that these data may be useful because we together with 27 other laboratories participate in a steroid profile scheme provided by the UK NEQAS (International Quality Expertise, [www.ukneqas.org.uk](http://www.ukneqas.org.uk)). In this scheme data are compared as described in the Methods section. This assures quality of the

analysis and confirms that there are ways to compare steroid data generated by different laboratories.

Limitations of our study are the rather small number of subjects (though one of the biggest to date) and urine collection mode. Although small in numbers, we studied our subjects longitudinally with short intervals, and are therefore able to provide statistically valid, calculated normative data. 24 hour urine collections in neonates and infants are cumbersome and mostly imprecise but provide real quantitative data of excreted metabolites, while spot urine sampling is easy to perform but requires calculation of excreted metabolites in relation to creatinine values. However, previous studies showed that spot urine measurements are valid for diagnostic purposes [30]. For group analysis, we have chosen to categorize the steroid metabolites according to their receptor binding. However, this choice left us with the problem that corticosterones bind to mineralocorticoid and glucocorticoid receptors which prompted us to put them in a separate group. Grouping of steroids is not standardized and may be performed differently according to study needs.

## **5 Conclusions**

The following conclusions about the urinary steroid metabolome in the first year of life emerge from our analyses. First, the creatinine corrected concentration of urinary excreted steroids is high in infants during the first two months of life with a peak around 3 weeks of life. Then these steroids decrease to one fifth by week 25 in both sexes. Second, the pattern of urinary steroid excretion in the first year of life does not run parallel in all steroid groups. Glucocorticoid, mineralocorticoid and corticosterone metabolites seem to be excreted rather at a constant level while progestins, androgens and estrogens decline markedly within the first few months of life. Third, the urinary steroid metabolome mirrors the underlying developmental biology of human steroidogenesis and is therefore a powerful tool for steroid research and diagnostics. However, detailed age- and sex-related normative values are essential.

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## 7 References

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**Legends to Figures and Tables:**

**Table 1: Steroid metabolites measured in this study and their molar mass.** The nomenclature of the systematic names follows the recommendations published by the IUPAC commission on the Nomenclature of Organic Chemistry in 1969 [32], amended by the IUPAC–IUB Commission on Biochemical Nomenclature in 1971 [33] and again revised in 1989 [34]. OH: hydroxy, DH: dihydro, TH: tetrahydro.

**Table 2. Baseline characteristics of the study subjects dichotomized by sex.**

**Table 3. Fifty-two urinary steroid metabolites at 13 time-points in the first year of life display no sex specificity.** Age and sex dependency of each log-transformed steroid has been estimated the described statistical models. Data are presented as 10<sup>th</sup>-50<sup>th</sup>-90<sup>th</sup> percentiles and expressed in the unit  $\mu\text{g} / \text{mmol Creatinine}$ . N represents the sample number per analyte included in the statistical model. The association with age effect is indicated.

**Table 4. Fifteen urinary steroid metabolites at 13 time-points in the first year of life display sex specificity.** Age and sex dependency of each log-transformed steroid has been estimated the described statistical models. Data are presented as 10<sup>th</sup>-50<sup>th</sup>-90<sup>th</sup> percentiles and expressed in the unit  $\mu\text{g} / \text{mmol creatinine}$ . The association with age effect is indicated. N represents the sample number per analyte, included in the statistical model. m: male, f: female

**Figure 1. Pathways of steroid hormone biosynthesis.** Pathways from multiple cell types are combined to provide an overview of all possible biosynthesis processes. The 67 steroid metabolites measured in this study are highlighted by colored background according to their suggested receptor activity. Enzyme activities are displayed in blue color. Abbreviations: DHEA: Dehydroepiandrosterone. The OH in enzyme names indicates a hydroxylase, e.g. 17 $\alpha$ -OH: 17 $\alpha$ -hydroxylase. The OH in steroid names indicates a hydroxyl group, e.g. 17-OH-pregnenolone: 17-hydroxy-pregnenolone. DH: dehydro; TH: tetrahydro; HSD: hydroxysteroid dehydrogenase; POR: P450 oxidoreductase; Cyt b5: cytochrom b5; 5 $\alpha$ -R: 5 $\alpha$ -reductase; 5 $\beta$ -R: 5 $\beta$ -reductase; CYP11B2: aldosterone synthase; C17,20-lyase2: yet undefined lyase, that removes the side-chain of 17-OH-corticosteroids [31].

**Figure 2. Urinary steroid excretion in  $\mu\text{g}/\text{mmol creatinine}$  by sex and age in the first year of life.** Note the logarithmic scaling of all Y-axes. A and B. Longitudinal comparison of the total urinary steroid excretion and the steroid excretion by steroid groups in boys (A) and girls (B). C-L. Estimated percentile curves for urinary steroid metabolites by sex and age.

The solid lines represent the 50th and the dashed lines the 3<sup>rd</sup>, 10<sup>th</sup>, 25<sup>th</sup>, 75<sup>th</sup>, 75<sup>th</sup>, 90<sup>th</sup>, and 97<sup>th</sup> percentiles as indicated based on the described statistical models.

**Table 1: Steroid metabolites measured in this study and their molar mass.** The nomenclature of the systematic names follows the recommendations published by the IUPAC commission on the Nomenclature of Organic Chemistry in 1969 [32], amended by the IUPAC–IUB Commission on Biochemical Nomenclature in 1971 [33] and again revised in 1989 [34]. OH: hydroxy, DH: dihydro, TH: tetrahydro.

Trivial name	Systematic name
<b>Progesterones</b>	
progesterone	4-pregnene-3,20-dione
6 $\alpha$ -OH-progesterone	6 $\alpha$ -hydroxy-4-pregnene-3,20-dione
6 $\beta$ -OH-progesterone	6 $\beta$ -hydroxy-4-pregnene-3,20-dione
11 $\alpha$ -OH-progesterone	11 $\alpha$ -hydroxy-4-pregnene-3,20-dione
11 $\beta$ -OH-progesterone	11 $\beta$ -hydroxy-4-pregnene-3,20-dione
11-keto-progesterone	4-pregnene-3,11,20-trione
17 $\alpha$ -OH-progesterone	17 $\alpha$ -hydroxy-4-pregnene-3,20-dione
17-OH-pregnanolone	3 $\beta$ ,17-dihydroxy-5-pregnen-20-one
pregnanetriol	5 $\beta$ -pregnane-3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -triol
pregnenetriol	5-pregnene-3 $\beta$ ,17 $\alpha$ ,20 $\alpha$ -triol
pregnanetriolone	3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ -trihydroxy-5 $\beta$ -pregnan-11-one
5 $\alpha$ -DH-progesterone	5 $\alpha$ -pregnane-3,20-dione
3 $\alpha$ 5 $\alpha$ -TH-progesterone / allopregnanolone	3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one
3 $\beta$ 5 $\alpha$ -TH-progesterone / isopregnanolone	3 $\beta$ -hydroxy-5 $\alpha$ -pregnan-20-one
5 $\beta$ -DH-progesterone	5 $\beta$ -pregnane-3,20-dione
3 $\alpha$ 5 $\beta$ -TH-progesterone / epipregnanolone	3 $\alpha$ -hydroxy-5 $\beta$ -pregnan-20-one
6 $\alpha$ -OH-3 $\alpha$ 5 $\beta$ -TH-progesterone	3 $\alpha$ ,6 $\alpha$ -dihydroxy-5 $\beta$ -pregnan-20-one
3 $\beta$ 5 $\beta$ -TH-progesterone / pregnanolone	3 $\beta$ -hydroxy-5 $\beta$ -pregnan-20-one
20 $\alpha$ -DH-progesterone	20 $\alpha$ -hydroxy-4-pregnen-3-one
17 $\alpha$ -OH-20 $\alpha$ -DH-progesterone	17 $\alpha$ ,20 $\alpha$ -dihydroxy-4-pregnene-3,20-dione
20 $\alpha$ -DH-5 $\alpha$ -DH-progesterone	20 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-3-one
20 $\alpha$ -DH-3 $\alpha$ 5 $\alpha$ -TH-progesterone / allopregnanediol	5 $\alpha$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol
20 $\alpha$ -DH-3 $\beta$ 5 $\alpha$ -TH-progesterone	5 $\alpha$ -pregnane-3 $\beta$ ,20 $\alpha$ -diol
20 $\alpha$ -DH-3 $\alpha$ 5 $\beta$ -TH-progesterone / pregnanediol	5 $\beta$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol
20 $\alpha$ -DH-3 $\beta$ 5 $\beta$ -TH-progesterone	5 $\beta$ -pregnane-3 $\beta$ ,20 $\alpha$ -diol
20 $\beta$ -DH-progesterone	20 $\beta$ -hydroxy-4-pregnen-3-one
<b>Androgens</b>	
dehydroepiandrosterone	3 $\beta$ -hydroxy-5-androsten-17-one
16 $\alpha$ -OH-dehydroepiandrosterone	3 $\beta$ ,16 $\alpha$ -dihydroxy-5-androsten-17-one
androstenediol	5-androstene-3 $\beta$ ,17 $\beta$ -diol
testosterone	17 $\beta$ -hydroxy-4-androsten-3-one
5 $\alpha$ -DH-testosterone	17 $\beta$ -hydroxy-5 $\alpha$ -androstan-3-one
androstanediol / dihydroandrosterone	5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol

Trivial name	Systematic name	M
androsterone	3 $\alpha$ -hydroxy-5 $\alpha$ -androstan-17-one	
5-androstenetriol	5-androstene-3 $\beta$ ,16 $\alpha$ ,17 $\beta$ -triol	
11 $\beta$ -OH-androsterone	3 $\alpha$ ,11 $\beta$ -dihydroxy-5 $\alpha$ -androstan-17-one	
etiocholanolone	3 $\alpha$ -Hydroxy-5 $\beta$ -androstan-17-one	
Estrogens		
17 $\beta$ -estradiol	1,3,5(10)-estratriene-3,17 $\beta$ -diol	
estriol	1,3,5(10)-estratriene-3,16 $\alpha$ ,17 $\beta$ -triol	
Corticosterones		
11-deoxycorticosterone	21-hydroxy-pregnene-3,20-dione	
11-deoxy-TH-corticosterone	3 $\alpha$ ,21-dihydroxy-5 $\beta$ -pregnan-20-one	
11-dehydro-TH-corticosterone	3 $\alpha$ ,21-dihydroxy-5 $\beta$ -pregnane-11,20-dione	
18-OH-11-dehydro-TH-corticosterone	3 $\alpha$ ,18,21-trihydroxy-5 $\beta$ -pregnane-11,20-dione	
TH-corticosterone	3 $\alpha$ ,11 $\beta$ ,21-trihydroxy-5 $\beta$ -pregnan-20-one	
allo-TH-corticosterone	3 $\alpha$ ,11 $\beta$ ,21-trihydroxy-5 $\alpha$ -pregnan-20-one	
Mineralocorticoids		
TH-aldosterone	11 $\beta$ ,18-epoxy-3 $\alpha$ ,18,21-trihydroxy-5 $\beta$ -pregnan-20-one	
TH-11-deoxycortisol	3 $\alpha$ ,17,21-trihydroxy-5 $\beta$ -pregnan-20-one	
Glucocorticoids		
cortisol	11 $\beta$ ,17,21-trihydroxy-4-pregnene-3,20-dione	
6 $\beta$ -OH-cortisol	6 $\beta$ ,11 $\beta$ ,17 $\alpha$ ,21-tetrahydroxy-4-pregnene-3,20-dione	
18-OH-cortisol	11,17,18,21-tetrahydroxy-4-pregnene-3,20-dione	
20 $\alpha$ -DH-cortisol	11 $\beta$ ,17,20 $\alpha$ ,21-tetrahydroxy-4-pregnen-3-one	
TH-cortisol	3 $\alpha$ ,11 $\beta$ ,17,21-tetrahydroxy-5 $\beta$ -pregnan-20-one	
$\alpha$ -cortol	5 $\beta$ -pregnane-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,20 $\alpha$ ,21-pentol	
$\beta$ -cortol	5 $\beta$ -pregnane-3 $\alpha$ ,11 $\beta$ ,17 $\alpha$ ,20 $\beta$ ,21-pentol	
11 $\beta$ -OH-etiocholanolone	3 $\alpha$ ,11 $\beta$ -dihydroxy-5 $\beta$ -androstan-17-one	
allo-TH-cortisol	3 $\alpha$ ,11 $\beta$ ,17,21-tetrahydroxy-5 $\alpha$ -pregnan-20-one	
cortisone	17,21-dihydroxy-4-pregnene-3,11,20-trione	
20 $\alpha$ -DH-cortisone	17 $\alpha$ ,20 $\alpha$ ,21-trihydroxy-4-pregnene-3,11-dione	
20 $\beta$ -DH-cortisone	17 $\alpha$ ,20 $\beta$ ,21-trihydroxy-4-pregnene-3,11-dione	
TH-cortisone	3 $\alpha$ ,17,21-trihydroxy-5 $\beta$ -pregnan-11,20-dione	
1 $\beta$ -OH-TH-cortisone	1 $\beta$ ,3 $\alpha$ ,17,21-tetrahydroxy-5 $\beta$ -pregnan-11,20-dione	
6 $\alpha$ -OH-TH-cortisone	3 $\alpha$ ,6,17,21-tetrahydroxy-5 $\beta$ -pregnan-11,20-dione	
$\alpha$ -cortolone	3 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ ,21-tetrahydroxy-5 $\beta$ -pregnane-11-one	
6 $\alpha$ -OH- $\alpha$ -cortolone	3 $\alpha$ ,6 $\alpha$ ,17 $\alpha$ ,20 $\alpha$ ,21-pentahydroxy-5 $\beta$ -pregnane-11-one	
$\beta$ -cortolone	3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ ,21-tetrahydroxy-5 $\beta$ -pregnane-11-one	
1 $\beta$ -OH- $\beta$ -cortolone	1 $\beta$ ,6 $\alpha$ ,17 $\alpha$ ,20 $\beta$ ,21-pentahydroxy-5 $\beta$ -pregnane-11-one	
6 $\alpha$ -OH- $\beta$ -cortolone	3 $\alpha$ ,6 $\alpha$ ,17 $\alpha$ ,20 $\beta$ ,21-pentahydroxy-5 $\beta$ -pregnane-11-one	
11-keto-etiocholanolone / 11-oxo-etiocholanolone	3 $\alpha$ -hydroxy-5 $\beta$ -androstane-11,17-dione	

Table 2 Baseline characteristics of the study subjects dichotomized by sex.

	Female	Male	All
n	22	21	43

	Female	Male	All
n	22	21	43
Vaginal delivery	16	15	31
Birth weight (g) - mean	3253	3281	3267
BW (g) - median	3400	3270	3390
BW (SDS)	-0.33	-0.58	-0.45
BW (g) range	2560-4000	2400-3860	2400-4000
Weight at 1 year (kg) - mean	9.19	9.86	9.52
W1y (kg) - median	8.9	9.69	9.5
W1y (mean - SD)	-0.34	-0.3	-0.32
W1y (kg) range	7.4-11.9	8.3-11.2	7.4-11.9

**Table 3. Fifty-two urinary steroid metabolites at 13 time-points in the first year of life display no sex specificity.** Age and sex dependency of each log-transformed steroid has been estimated the described statistical models. Data are presented as 10<sup>th</sup>-50<sup>th</sup>-90<sup>th</sup> percentiles and expressed in the unit  $\mu\text{g} / \text{mmol Creatinine}$ . N represents the sample number per analyte included in the statistical model. The association with age effect is indicated.

Metabolite	N	Week 1 10 <sup>th</sup> -50 <sup>th</sup> -90 <sup>th</sup>	Week 3 10 <sup>th</sup> -50 <sup>th</sup> -90 <sup>th</sup>	Week 5 10 <sup>th</sup> -50 <sup>th</sup> -90 <sup>th</sup>	Week 7 10 <sup>th</sup> -50 <sup>th</sup> -90 <sup>th</sup>	Week 9 10 <sup>th</sup> -50 <sup>th</sup> -90 <sup>th</sup>	Week 11 10 <sup>th</sup> -50 <sup>th</sup> -90 <sup>th</sup>	Week 13 10 <sup>th</sup> -50 <sup>th</sup> -90 <sup>th</sup>	Week 17 10 <sup>th</sup> -50 <sup>th</sup> -90 <sup>th</sup>
6 $\alpha$ -OH-progesterone	362	35-312-2073	27-242-1609	22-190-1264	17-151-1004	14-121-806	11-98-655	9-81-5386	56-3752
6 $\beta$ -OH-progesterone	360	2-11-1081	1-8-82	1-7-64	1-5-50	1-4-39	0-3-31	0-3-26	0-2-17
17 $\alpha$ -OH-progesterone	361	20-65-175	16-52-141	13-42-114	11-34-939	9-28-76	7-23-63	6-19-53	4-14-38
17-OH-pregnanolone	424	3-10-30	2-10-28	2-9-26	2-8-24	2-8-23	2-7-22	2-7-21	2-7-19
pregnanetriol	424	5-14-39	7-22-60	7-22-60	7-22-60	7-22-60	7-22-60	7-22-60	7-22-60
pregnenetriol	424	4-15-58	7-28-109	10-40-156	12-45-173	10-40-153	9-34-1328	30-1156	23-89
pregnanetriolone	424	1-4-12	1-4-12	1-3-11	1-3-11	1-3-10	1-3-10	1-3-9	1-3-9
5 $\alpha$ -DH-progesterone	361	4-23-1185	5-31-1596	6-39-1957	7-44-2207	4-45-2287	7-43-2166	6-39-1965	3-33-1654
3 $\alpha$ 5 $\alpha$ -TH-progesterone	361	10-1-4	1-2-5	1-2-5	1-2-6	1-2-6	1-2-7	1-2-7	1-2-7
3 $\beta$ 5 $\alpha$ -TH-progesterone	362	1-3-10	1-4-11	1-4-11	1-4-11	1-3-10	1-3-9	1-2-7	0-2-5
3 $\alpha$ 5 $\beta$ -TH-progesterone	361	10-1-5	0-1-4	0-1-4	0-1-3	0-1-3	0-1-3	0-1-3	0-1-2
3 $\beta$ 5 $\beta$ -TH-progesterone	362	0-3-12	0-4-16	0-5-20	1-6-25	1-7-28	1-8-30	1-8-30	1-8-30
20 $\alpha$ -DH-progesterone	360	9-127-514	4-60-2432	3-31-1261	1-18-71	1-11-44	1-7-29	0-5-22	0-4-15
17 $\alpha$ -OH-20 $\alpha$ -DH-progesterone	362	1-9-52	1-9-52	1-9-52	1-9-52	1-9-52	1-9-52	1-9-52	1-9-52
20 $\alpha$ DH3 $\alpha$ 5 $\alpha$ THprogesterone	362	2-13-42	2-10-35	1-9-29	1-7-25	1-6-21	1-5-19	1-5-16	1-4-13
20 $\alpha$ DH3 $\beta$ 5 $\alpha$ THprogesterone	361	1-4-28	1-4-25	1-3-22	1-3-19	0-3-17	0-2-16	0-2-14	0-2-12
20 $\alpha$ -DH-3 $\alpha$ 5 $\beta$ -TH-progesterone	422	1-9-39	1-7-28	1-5-23	1-5-20	1-5-20	1-5-20	1-5-20	1-5-20
20 $\alpha$ -DH-3 $\beta$ 5 $\beta$ -TH-progesterone	362	1-7-89	2-9-110	2-9-115	1-8-103	1-7-87	1-6-74	1-5-63	1-4-49
20 $\beta$ -DH-progesterone	362	9-92-3896	6-64-2724	4-46-1933	3-33-1402	2-25-1042	1-19-78	1-14-60	1-9-38
dehydroepiandrosterone	424	2-9-38	2-7-32	2-6-27	2-5-23	1-5-20	1-4-18	1-4-16	1-3-13

16 $\alpha$ -OH-dehydroepiandrosterone	136-4241088-8720	123-985-7898	94-757-6070	62-494-3959	38-301-2414	24-189-1515	15-122-979	7-56-4462
androstenediol	4248-29-1317	25-1106	21-94	5-18-80	4-15-69	4-13-60	3-12-52	3-9-41
testosterone	4241-7-60	1-5-48	1-4-39	1-3-32	0-3-27	0-2-23	0-2-19	0-2-15
androsterone	4244-10-27	5-14-38	6-15-40	5-13-36	4-12-32	4-11-29	4-10-27	3-8-23
5-androstenetriol	395-4241656-6005	385-1612-5846	329-1376-4990	246-1030-3735	165-689-2499	110-462-1676	76-317-1148	38-159-575
11 $\beta$ -OH-androsterone	4242-6-18	4-9-28	5-12-40	7-17-54	9-21-67	10-24-78	11-26-84	11-26-84
etiocholanolone	4241-3-15	1-3-13	1-3-12	1-2-11	1-2-10	1-2-10	1-2-10	1-2-10
estradiol	4210-1-4	0-1-3	0-1-3	0-1-3	0-1-3	0-1-2	0-1-2	0-0-2
estriol	42423-120-510	7-35-1494	19-80	2-12-49	1-7-31	1-5-20	1-3-14	0-2-7
11-dehydro-TH-corticosterone	42016-81-1265	19-98-1531	22-112-1754	24-121-1901	25-124-1950	24-121-1894	22-111-1740	17-85-1338
TH-corticosterone	4248-26-71	8-25-68	8-24-65	7-23-63	7-22-60	7-22-58	7-21-57	6-20-54
allo-TH-corticosterone	4235-28-1092	9-35	2-10-39	3-14-53	3-18-69	4-23-90	5-30-1158	46-1782
TH-aldosterone	4235-22-89	5-22-89	5-22-89	5-22-89	5-22-89	5-22-89	5-22-89	5-22-89
TH-11-deoxycortisol	4246-20-51	4-13-33	3-11-27	3-10-26	3-10-26	3-10-26	3-10-26	3-10-26
cortisol	42420-53-127	18-46-110	15-40-9614	35-8412	31-7511	28-6710	25-618	21-517
6 $\beta$ -OH-cortisol	4210-3-27	0-1-14	0-1-15	0-2-17	0-2-19	0-2-22	0-2-24	0-3-29
20 $\alpha$ -DH-cortisol	4210-1-6	0-1-6	0-1-6	0-1-6	0-1-6	0-1-7	0-1-7	0-1-9
TH-cortisol	4242-5-15	2-6-17	3-7-21	3-9-27	5-12-36	6-16-48	8-20-61	12-32-97
$\alpha$ -cortol	42416-49-145	23-74-216	27-85-249	25-79-232	23-72-210	21-66-192	19-60-177	17-52-153
$\beta$ -cortol	4244-11-24	6-15-35	8-21-47	10-27-6013	33-7315	38-8516	42-9416	17-44-100
11 $\beta$ -OH-etiocholanolone	4243-15-1113	19-1424	22-1594	21-1583	20-1473	19-1373	17-1293	16-114
cortisone	42424-83-194	24-84-197	23-82-192	22-77-180	20-69-162	17-61-141	15-53-124	12-42-98
20 $\alpha$ -DH-cortisone	4246-22-65	6-24-69	6-24-71	6-24-69	6-22-64	5-20-58	4-17-50	3-13-37
20 $\beta$ -DH-cortisone	4244-13-32	6-17-43	7-20-52	7-22-55	7-21-52	6-18-46	6-16-41	5-14-34
TH-cortisone	424186-536-1328	208-601-1488	226-652-1616	238-688-1703	244-703-1741	241-697-1726	232-670-1659	204-588-1456
1 $\beta$ -OH-TH-cortisone	40549-152-412	42-130-352	36-112-305	32-98-266	28-86-235	25-77-210	23-70-190	19-59-161
6 $\alpha$ -OH-TH-cortisone	942-4052679-6011	987-2805-6294	984-2798-6279	936-2660-5968	847-2408-5405	731-2078-4662	618-1758-3945	456-1295-2906
$\alpha$ -cortolone	42414-36-89	34-89-221	46-120-299	46-120-299	46-120-299	46-120-299	46-120-299	46-120-299
$\beta$ -cortolone	42471-200-449	113-316-709	150-420-942	167-470-1053	157-442-991	136-383-859	119-335-750	93-261-586
1 $\beta$ -OH- $\beta$ -cortolone	405106-413-1405	69-268-911	46-179-609	32-124-421	23-88-301	17-65-222	13-50-169	8-32-108

6 $\alpha$ -OH- $\beta$ -cortolone	417- 4051089- 3139	340-888-273-712-215-561-166-434-127-331-96-252-59-153-3	2560	2052	1617	1252	953	725	442	2
11-keto-etiocholanolone	4248-43-1938-40-1797-37-1677-35-1566-33-1476-31-1386-29-1305-26-118									

**Table 4. Fifteen urinary steroid metabolites at 13 time-points in the first year of life display sex specificity.** Age and sex dependency of each log-transformed steroid has been estimated the described statistical models. Data are presented as 10<sup>th</sup>-50<sup>th</sup>-90<sup>th</sup> percentiles and expressed in the unit  $\mu\text{g} / \text{mmol creatinine}$ . The association with age effect is indicated. N represents the sample number per analyte, included in the statistical model. m: male, f: female

Metabolite	sex	N	Week 1 10 <sup>th</sup> -50 <sup>th</sup> - 90 <sup>th</sup>	Week 3 10 <sup>th</sup> -50 <sup>th</sup> - 90 <sup>th</sup>	Week 5 10 <sup>th</sup> -50 <sup>th</sup> - 90 <sup>th</sup>	Week 7 10 <sup>th</sup> -50 <sup>th</sup> - 90 <sup>th</sup>	Week 9 10 <sup>th</sup> -50 <sup>th</sup> - 90 <sup>th</sup>	Week 11 10 <sup>th</sup> -50 <sup>th</sup> - 90 <sup>th</sup>	Week 13 10 <sup>th</sup> -50 <sup>th</sup> - 90 <sup>th</sup>	Week 15 10 <sup>th</sup> -50 <sup>th</sup> - 90 <sup>th</sup>
progesterone	m	1663-290-1865	2-198-1274	1-139-896	1-101-649	1-75-484	1-58-372	1-46-294	0-31-	
	f	1961-119-768	1-82-525	1-57-369	0-42-267	0-31-199	0-24-153	0-19-121	0-13-	
11 $\alpha$ -OH-progesterone	m	1662-139-648	2-122-566	2-107-499	1-95-443	1-85-397	1-77-359	1-70-328	1-60-	
	f	1961-63-291	1-55-254	1-48-224	1-43-199	1-38-178	1-35-161	1-32-147	0-27-	
11 $\beta$ -OH-progesterone	m	1651-11-124	1-11-124	1-11-124	1-11-124	1-11-124	1-11-124	1-11-124	1-11-	
	f	1950-5-57	0-5-57	0-5-57	0-5-57	0-5-57	0-5-57	0-5-57	0-5-5	
11-keto-progesterone	m	1661-59-4062	1-47-3234	1-38-2606	0-31-2125	0-25-1754	0-21-1465	0-18-1239	0-13-	
	f	1962-113-7801	1-90-6211	1-72-5005	1-59-4081	1-49-3369	1-41-2814	1-34-2379	0-26-	
5 $\beta$ -DH-progesterone	m	1662-21-169	2-16-129	1-12-101	1-10-80	1-8-65	1-7-53	1-6-45	1-4-3	
	f	1961-10-79	1-7-61	1-6-47	1-5-37	0-4-30	0-3-25	0-3-21	0-2-1	
6 $\alpha$ -OH-3 $\alpha$ 5 $\beta$ -TH-progesterone	m	16640-162-718	28-114-504	20-82-363	15-61-270	12-46-206	9-37-162	7-30-131	5-21-	
	f	19672-292-1294	51-205-908	37-148-655	27-110-486	21-84-371	16-66-292	13-53-236	9-38-	
20 $\alpha$ -DH-5 $\alpha$ -DH-progesterone	m	16681-25650-136837	44-13883-74063	25-7818-41707	15-4580-24435	9-2792-14894	6-1771-9445	4-1168-6232	2-573-3055	
	f	19626-8189-43682	14-4432-23644	8-2496-13314	5-1462-7801	3-891-4755	2-565-3015	1-373-1990	1-183-	
5 $\alpha$ -DH-testosterone	m	1963-27-185	4-33-226	5-38-258	5-40-274	5-40-272	4-37-251	4-32-219	3-25-	
	f	2282-15-101	2-18-124	2-21-141	3-22-150	3-22-148	2-20-137	2-18-120	2-13-	
androstanediol	m	1952-44-452	2-44-452	2-44-452	2-44-452	2-44-452	2-44-452	2-44-452	2-44-	
	f	2281-20-210	1-20-210	1-20-210	1-20-210	1-20-210	1-20-210	1-20-210	1-20-	
Deoxy-corticosterone	m	1663-38-216	1-20-113	1-13-77	1-12-67	1-12-67	1-12-67	1-12-67	1-12-	
	f	1961-15-88	1-8-46	0-6-31	0-5-28	0-5-28	0-5-28	0-5-28	0-5-2	
11-deoxy-TH-corticosterone	m	1952-25-626	2-21-514	2-17-427	1-14-359	1-12-305	1-11-263	1-9-229	1-7-1	
	f	2281-9-233	1-8-191	1-6-159	1-5-133	0-5-113	0-4-98	0-3-85	0-3-6	
18-OH-11-DH-TH-corticosterone	m	1960-5-147	0-5-147	0-5-147	0-5-147	0-5-147	0-5-147	0-5-147	0-5-1	
	f	2280-2-74	0-2-74	0-2-74	0-2-74	0-2-74	0-2-74	0-2-74	0-2-7	
18-OH-cortisol	m	1930-1-568	0-1-568	0-1-568	0-1-568	0-1-568	0-1-568	0-1-568	0-1-5	
	f	2260-1-296	0-1-296	0-1-296	0-1-296	0-1-296	0-1-296	0-1-296	0-1-2	
allo-TH-cortisol	m	1961-5-14	3-10-31	6-21-63	12-39-120	20-70-211	33-114-346	51-173-526	93-31-966	
	f	2281-3-8	2-6-17	3-11-35	6-22-66	11-39-117	19-63-192	28-96-291	52-17	

		Week 1	Week 3	Week 5	Week 7	Week 9	Week 11	Week 13	Week 15
m	178	2832-7636-18456	3365-9073-21927	3740-10085-24373	3890-10488-25347	3784-10204-24662	3445-9289-22449	3044-8208-19836	2428-6546-15819
	227	1914-5162-12475	2274-6132-14821	2528-6816-16474	2629-7089-17132	2558-6897-16669	2328-6278-15174	2057-5548-13408	1641-4424-10693
6 $\alpha$ -OH- $\alpha$ -cortolone									

Fig. 1

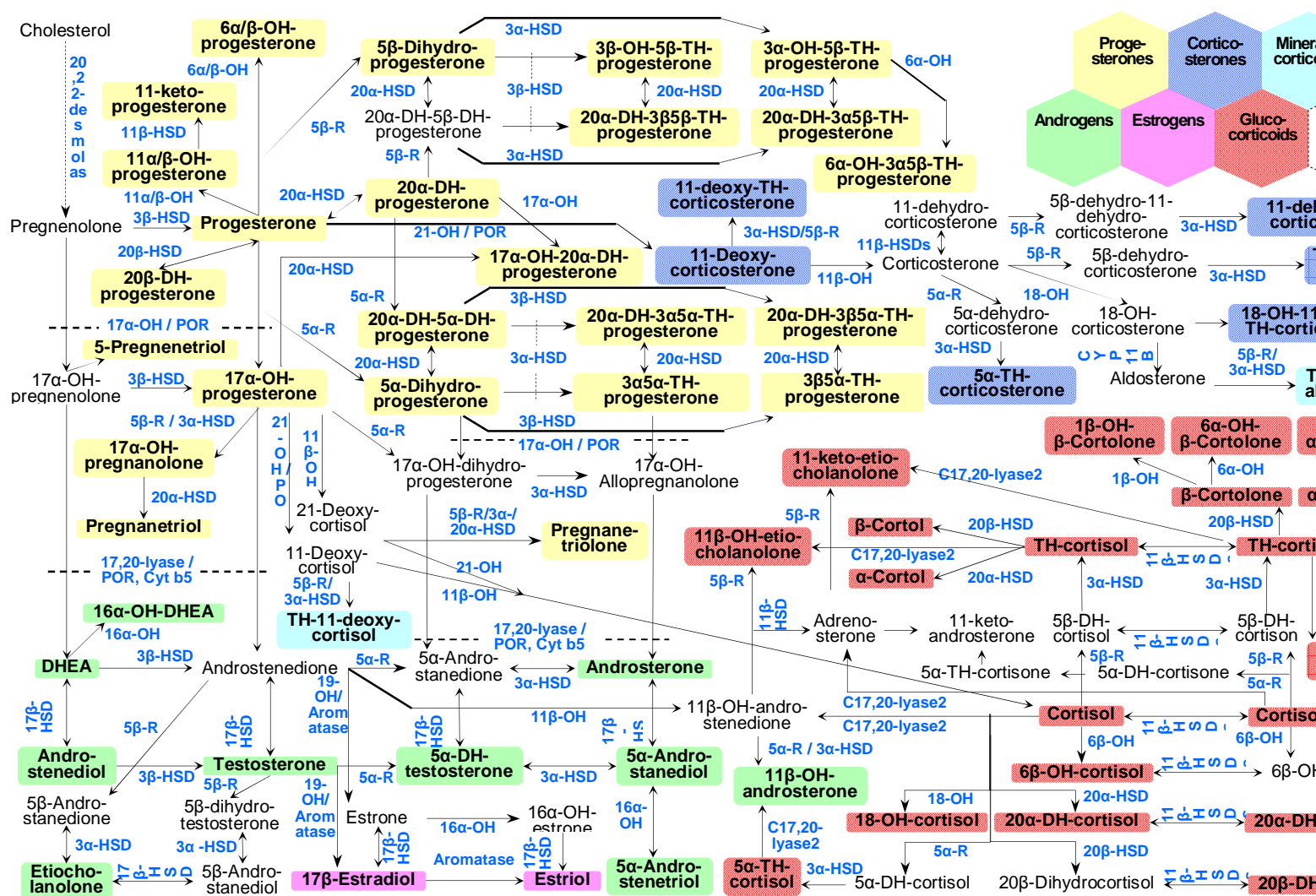


Fig. 2



